Primary Thrombophilia in México XI: Activated Protein C Resistance Phenotypes are Multifactorial

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Abstract Activated protein C resistance (aPCR) phenotypes represent around 20% of the laboratory findings in Mexican Mestizos having suffered thrombosis and displaying clinical markers of thrombophilia. In a single institution for a 276-month period, 96 Mexican mestizos with a history of thrombosis and clinical markers of a primary thrombophilic state were prospectively studied to identify a thrombophilic condition. An abnormal aPCR phenotype was identified in 18 individuals. Evaluation of those with an abnormal aPCR phenotype, identified that 44% had factor V Leiden mutation, 22% increased levels of factor VIII, 16% anti-phospholipid antibodies and 6% a lupus anticoagulant. In the remaining 22%, the use of direct oral anticoagulants (DOACs) in the past period of 12–24 h was recorded. We found significant associations between abnormal aPCR phenotype and the factor V Leiden mutation ($p = 0.022$), between abnormal aPCR phenotype and the use of DOACs ($p = 0.006$) and between antiphospholipid antibodies and lupus anticoagulant ($p < 0.0001$). These data are consonant with those observed in other populations and further identify that consideration be given to identifying whether individuals are being treated with the novel DOACs when conducting laboratory studies oriented to identify the etiology of thrombosis.

Keywords Thrombophilia · Protein C · Resistance

Introduction

Since 1999 we have been studying and treating patients with suspected inherited and/or acquired thrombophilia [1–3]. With a full laboratory workup, we are now able to identify inherited and acquired thrombophilic conditions in more than 90% of individuals having suffered thrombosis and displaying clinical markers of thrombophilia. As a result of studying these conditions in more than 500 Mexican mestizos, we have been able to define the thrombophilic conditions prevailing in the Mexican population, which express differences to those identified in other populations of the world [4].

The activated protein C resistance (aPCR) phenotype may be the result of either inherited or acquired conditions and it is present in 20% of the Mexican Mestizos having suffered thrombosis and having clinical markers of thrombophilia [2–4]. In an effort to gain further insight in the analysis of the causes of thrombophilia in México, we analyze herein the etiology of the abnormal aPCR phenotype identified in a subset of 18 individuals accrued within a group of 96 Mexican Mestizo individuals who were prospectively studied as a result of displaying a clinical marker of primary thrombophilia.

Materials and Methods

Patients

During a 276-month period, all consecutive Mexican mestizo patients referred to our clinic by physicians from

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different parts of the country were prospectively accrued in the study, if they had one of the following clinical markers associated with a primary hypercoagulable state [1–4]: (a) thrombosis at age younger than 40 years; (b) family history of thrombosis (first degree relatives); (c) recurrent thrombosis without apparent precipitating factors; (d) thrombosis at unusual anatomic sites and (e) resistance to conventional antithrombotic therapy. Individuals with overt malignancy, pregnancy, puerperium, oral contraceptives, post-surgery period, prolonged immobilization or other conditions associated with secondary thrombophilia were excluded from the study. All individuals had experienced at least one episode of venous thrombosis, confirmed by either phlebography or Doppler in the previous 30 days, and were not receiving either oral anticoagulants or antiplatelet drugs in the previous 12 h; no surgery nor trauma patients were included. The operational definition of mestizo by Pons-Estel et al. [5] was employed to select the patients: Individuals born in Latin America who had both Amerindian and white ancestors. Informed consent was obtained from all the individuals studied; the study was approved by the ethics and human research subject protection committees of the Clínica RUIZ de Puebla. This study is part of the prospective study which we have conducted to analyze the salient features of the thrombophilic conditions in Mexican mestizos.

Analytical Methods

(a) Assessment of Sticky Platelet Syndrome (SPS): The method described by Mammen [6] was used: blood was drawn, usually between 8:30 and 10:30 am, by clean venipuncture using 19 or 21 gauge butterfly needles. After venipuncture the tourniquet was released and the first 5 ml discarded. Then, 18 ml of blood was aspirated into a 20-ml syringe containing 2 ml of 3.8% sodium citrate solution. The anticoagulated blood was centrifuged as soon as possible for 10 min at 100g at room temperature to obtain platelet-rich plasma (PRP). About one-half of this PRP was recentrifuged at 2000g for 20 min at room temperature to obtain platelet-poor plasma (PPP). For aggregation, the PRP was diluted with the PPP to give a platelet count of $200 \times 10^9/L$. Platelet aggregation was measured in an aggregometer (Model 500 CA, ChronoLog Corporation, Havertown, PA, U.S.A.), employing the technique originally described by Born and Cross [7]. Changes in optical density were recorded on a Chrono Log recorder (model 703). While keeping temperature (37 °C) and stirrer speed constant, aggregation was induced by three concentrations of ADP (2.34, 1.17, and 0.58 μM), and by three concentration of epinephrine (11, 1.1, and 0.55 μM), (final concentration in the PRP cuvette). Maximal aggregation was expressed as percentage of 100% light transmission,

calibrated for each specimen. Normal controls were studied for each case. Abnormal results for platelet aggregation with three concentrations of ADP (2.34, 1.17, and 0.58 μM) were found to be above 55, 36 and 12%, whereas for the three concentrations of epinephrine (11, 1.1, and 0.55 μM) were above 80, 27 and 20% [4].

(b) The activated protein C resistance (aPCR) phenotype was measured using the test kit ProC global (Siemens Healthacre Diagnostic Products GmbH, Marburg, Germany): aPCR was determined by measuring the prolongation of activated partial thromboplastin time in response to aPC, employing factor V deficient plasma [1].

(c) Coagulation protein C activity and antigen, coagulation protein S, antithrombin III, plasminogen, tissue-type plasminogen activator activity, plasminogen activator inhibitor activity, plasminogen activator inhibitor type, IgG and IgM isotypes of anti-phospholipid antibodies and lupus anticoagulants (LA) were assessed as previously described [1].

(d) Factor V gene mutations: A polymerase chain reaction (PCR)-based analysis for the factor V p.506R > Q (Leiden) gene mutation was performed according to Zöller and Dahlbäck [8]. Briefly, a region of the factor V gene comprising nucleotides 1690–1692 of the codon 506 was amplified by PCR and then subjected to digestions with restriction endonuclease Mnl I. The restriction pattern was analyzed by electrophoresis on a 4.5% polyacrylamide gel. The oligonucleotides used are described in reference 3. To look for the factor V HR2 haplotype, the exon 13 was amplified with the primer pair F5 13-1 and F5 13-2 [9]. The polymorphism p.1299H > R which forms part of the allele R2 was then detected by restriction analysis with RsaI [9]. For the detection of the p.306R > T (Cambridge) mutation, the exon 7 of the factor V gene was amplified using the primers F5 7-1 and F5 7-2 [10]. The mutation was identified by restriction analysis with BstNI. To look for the factor V gene Hong Kong mutation (p.306R > G), the 241 bp amplification product obtained for the detection of the factor V Cambridge mutation was digested with HpaII according to Chan et al. [11]. To identify the factor V gene Liverpool mutation (p.359I > T), the exon 8 of the factor V gene was amplified using the primers F5 8-1 and F5 8-2 [9]. The amplification product obtained for the detection of the FV Liverpool mutation was digested with BsrI according to Mumford et al. [12].

(e) c.677C > T mutation in the 5,10-methylen-tetrahydrofolate-reductase (MTHFR): The detection of the transition c.677C > T of the MTHFR gene was essentially performed as described in Kluijtmans et al. [13]. A 108 bp fragment of the MTHFR gene comprising nucleotides 598–705 was amplified. In order to determine the genotype the amplicon was subsequently subjected to digestion using HinfI.

(f) c.20210G > A polymorphism in the 3'-untranslated region of the prothrombin gene: A PCR-based analysis for this gene mutation was performed essentially as described by Poort et al. [14]. Briefly, a fragment of the prothrombin gene comprising nucleotides 19,889–20,212 was amplified by PCR. One of the primers introduces a mutation which together with the G > A mutation generates a HindIII restriction site. An aliquot of the amplified product was therefore subjected to digestion with the restriction endonuclease HindIII.

Data Analysis

The highly efficient algorithm C4.5 for data mining as implemented and improved in the application See5 [15, 16] was used to analyze the data of this study. As the number of cases in our study was small, the complete data set was randomly split into 10 subsets with approximately equal distribution of classes and number of cases. Each case is defined by the values of the attributes (sticky platelet syndrome, FV Leiden, Cambridge, Hong Kong and Liverpool mutations, c.677C > T mutation in the MTHFR gene, c.20210G > A mutation in the 3'-untranslated region of the prothrombin gene, FVIII levels, antiphospholipid antibodies, protein C levels, protein S levels, lupus anticoagulants, activated protein C resistance phenotype and use of DOACs). After selecting one attribute as target attribute the remaining attributes were treated as predictors. A decision tree was constructed using nine subsets, expressed as an easily interpretable rule sets, and cross-validated with the hold-out subset. The accuracy of the classification was recorded. This process was repeated permutating the attributes until each attribute had been selected once as target attribute. Cohen's kappa was used to determine the reliability of the rule set [17].

Results

Of the 96 consecutive patients that were accrued in the study, 32 (33%) were males, the median age being 39 years (range 5–73). In 18 persons (19%), an abnormal aPCR phenotype was identified. Of these, 44% were found to have the factor V Leiden mutation, 22% increased levels of factor VIII, 16% anti-phospholipid antibodies and 6% a lupus anticoagulant. Interestingly, in the remaining 22% of these patients with an abnormal aPCR phenotype, the use of direct oral anticoagulants (DOACs) in the past period of 12–24 h was recorded. Seven patients with abnormal aPCR phenotype had both exposure to DOACs and one of the above mentioned causes of an abnormal aPCR; all of them were retested, finding persistence of the abnormal aPCR phenotype, showing that it was not due to the use of

DOAC's. Accordingly, in 11 patients (61%) with an abnormal aPCR phenotype, the recent use of DOACs was recorded as the single cause of the abnormality, see Table 2.

By means of the χ^2 test [15–17], we found significant associations between an abnormal aPCR phenotype and the recent use of DOACs, and the presence of the factor V Leiden mutation, see Table 1. We also looked for the association between the abnormal aPCR and the identification of the sticky platelet syndrome phenotype, which was non significant ($p = 0.34$). We further identified additional causes of abnormal aPCR phenotype: Increased factor VIII levels, presence of antiphospholipid antibodies (APLA) and lupus anticoagulant (LA); however, their proportion was not different from that observed in thrombophilic individuals not displaying the aPCR phenotype (Table 1). The rest of the variables studied were not found to be associated with the presence of the aPCR phenotype.

When using the See5 data mining application, four rule sets were found yielding contingency tables significantly distinct from an expected distribution as determined by Fisher's exact test ($p < 0.05$: prediction of activated protein C resistance, lupus anticoagulants, antiphospholipid antibodies and use of DOACs). However, none of them were sufficiently accurate according to Cohen's kappa (values between 0.25 and 0.51), mostly due to bad sensitivities (ranging between 30 and 50%), while the specificities were rather good (89–100%). Interestingly, when looking at the rule sets for aPCR phenotype, in all cases only the status of FV Leiden mutation had been considered relevant for the classification. Similarly, presence of antiphospholipid antibodies was predicted only by the presence of lupus anticoagulants and vice versa. Finally, use of DOACs was conditioned exclusively by the presence of activated protein C resistance.

By employing the odds ratio method [15–17] among all the variables studied, we found highly significant associations between abnormal aPCR phenotype and the factor V

Table 1 Salient findings in thrombophilic patients with abnormal or normal activated protein C resistance phenotype (aPCR)

	Abnormal aPCR (n = 18) %	Normal aPCR (n = 78) %	<i>p</i> value
DOACs	61	28	0.01
Factor V Leiden	44	0	0.01
Increased F VIII	22	24	0.96
APLA	16	12	0.74
LA	6	16	0.57

DOACs Direct oral anticoagulants, APLA Antiphospholipid antibodies, LA Lupus anticoagulant

p values have been obtained by the χ^2 method

Table 2 Significant associations obtained by means of the odds ratio test

	Odds ratio	<i>p</i> value
aPCR/FV Leiden	97.8	0.0022
aPCR/DOACs	4.7	0.0065
APLA/LA	29.8	0.0001

aPCR activated protein C resistance phenotype, *DOACs* Direct oral anticoagulants, *APLA* Antiphospholipid antibodies, *LA* Lupus anticoagulant

Leiden mutation ($p = 0.0022$), between abnormal aPCR phenotype and the use of DOACs ($p = 0.006$) and also between antiphospholipid antibodies and lupus anticoagulant ($p < 0.0001$) (Table 2). We also observed increased factor VIII levels, presence of antiphospholipid antibodies and lupus anticoagulant as a cause of abnormal aPCR phenotype but, again, their prevalence was not significantly different from that observed in thrombophilic individuals not displaying the aPCR phenotype (Table 2).

Discussion

Proteolytic inactivation of factors Va (FVa) and VIIIa (FVIIIa) by activated protein C (aPC) and its cofactors protein S and factor V (FV) is a key process in the physiological down-regulation of blood coagulation [3]. Functional abnormalities of this pathway, which manifest itself in vitro as a poor anticoagulant response of plasma to added APC (aPC resistance, aPCR) are prevalent in the general population and are associated with an increased risk of venous thrombosis. aPCR was originally discovered in thrombophilic families and later shown to be associated with the common FV p.506R > Q (FV Leiden) mutation, which abolishes one of the aPC-cleavage sites in FV [22]. Although FV Leiden is the major cause of hereditary aPCR, it is becoming increasingly clear that several other genetic and acquired conditions contribute to aPCR and thereby increase the risk of venous thrombosis [18–21]. The factor V Cambridge, Hong Kong and Liverpool mutations can also lead into the aPCR phenotype [2, 18], as can the presence of LA and/or antiphospholipid antibodies [18, 19], increased factor VIII levels [18, 20], dysfunctional protein S molecules, various hormone therapies including oral contraceptive use [21], cancer [20] and the use of DOACs [22–24].

The C4.5 machine learning algorithm did not reveal any hidden associations between the case attributes but rather expected ones: FV Leiden mutation predicted aPCR phenotype which by itself predicted the use of DOACs. Furthermore, autoimmune diseases predicted each other, i.e. the presence of antiphospholipid antibodies predicted the

presence of lupus anticoagulants and vice versa. Interestingly, each rule set consisted in all the mentioned cases of just one candidate predictor.

We have previously shown that an abnormal aPCR phenotype is recorded in around 20% of Mexican mestizo patients with clinical markers of primary thrombophilia [2–4]. In this study we have analyzed some of the causes, both inherited and acquired, of the aPCR phenotype in a group of Mexican mestizos with a history of a thrombotic episode in the presence of a clinical marker of thrombophilia. We have found that the most frequent cause of the aPCR phenotype is the presence of the FV Leiden mutation, followed by increased levels of FVIII, APLA and LA. Interestingly, in 61% of these patients we were able to record recent use of DOACs, in 39% of cases associated with an additional cause of an abnormal aPCR phenotype and in the rest of the instances as the single possible explanation of the abnormal phenotype. These data are consistent with those observed in other populations and identify that consideration be given as to whether individuals are being treated with the novel DOACs when conducting laboratory studies oriented to identify the etiology of thrombosis [24]. Additional studies are needed to define with more detail the etiology of the aPCR phenotype and its association with a thrombosis-prone condition.

Compliance with Ethical Standards

Conflict of interest The authors declare no conflict of interest.

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